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## Limited heterogeneity of rearranged T-cell receptor V $\alpha$ transcripts in brains of multiple sclerosis patients

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THE identification of activated T cells in the brain of individuals with multiple sclerosis (MS) indicates that these cells are critical in the pathogenesis of this disease. In an attempt to elucidate the nature of the lymphocytic infiltration, we used the polymerase chain reaction to amplify T-cell antigen receptor (TCR) V $\alpha$  sequences from transcripts derived from MS brain lesions. In each of three MS brains, only two to four rearranged TCR V $\alpha$  transcripts were detected. No V $\alpha$  transcripts could be found in control brains. Sequence analysis of transcripts encoded by the V $\alpha$  12.1 region showed rearrangements to a limited number of J $\alpha$  region segments. These results imply that TCR V $\alpha$  gene expression in MS brain lesions is restricted.

Multiple sclerosis is an inflammatory disease of the central nervous system (CNS), characterized by myelin destruction<sup>1-3</sup>. In the brain, there is an accumulation of macrophages, plasma cells, major histocompatibility complex (MHC) class II positive antigen-presenting cells and activated cytokine-secreting T cells<sup>4-8</sup>. Several lines of evidence indicate that T lymphocytes migrate from the peripheral blood to the CNS compartment and participate directly in the formation of brain lesions<sup>9-11</sup>. There is also evidence of oligoclonality in T lymphocytes within the cerebrospinal fluid of MS patients<sup>12</sup>. In addition, TCR V $\alpha$  and V $\beta$  genes have been shown to contribute to the genetic control of susceptibility to this disease<sup>13-15</sup>.

To examine the expression of TCR genes at the site of disease, messenger RNA isolated from demyelinating brain plaques from three MS patients with chronic progressive disease, and from three control brains (non-MS) was used to synthesize complementary DNA. These cDNAs were then subjected to enzymatic gene amplification by the polymerase chain reaction (PCR) method<sup>16,17</sup> using specific TCR primers. The results of such an amplification using primers for the TCR V $\alpha$  12.1 family

TABLE 1 T-cell receptor  $\alpha$ -primers

| Primer        | Clone  | Sequence                         | Family members |
|---------------|--------|----------------------------------|----------------|
| V $\alpha$ 1  | HAP 10 | 5'-CTGAGGTGCACTACTCA-3'          | 1.1, 1.2, 1.3  |
| V $\alpha$ 2  | HAP 26 | 5'-GTGTTCCAGAGGGAGCCATTGCC-3'    | 2.1, 2.2       |
| V $\alpha$ 3  | HAP 05 | 5'-GGTGAACAGTCAACAGGGAGA-3'      | 3.1            |
| V $\alpha$ 4  | HAP 08 | 5'-ACAAGCATTACTGTACTCTCA-3'      | 4.1            |
| V $\alpha$ 5  | HAP 35 | 5'-GGCCCTGAACATTACGGA-3'         | 5.1            |
| V $\alpha$ 6  | HAP 01 | 5'-GTCACTTTCTAGCCTGCTGA-3'       | 6.1            |
| V $\alpha$ 7  | HAP 21 | 5'-AGGAGCCATTGTCCAGATAAA-3'      | 7.1, 7.2       |
| V $\alpha$ 8  | HAP 41 | 5'-GGAGAGAATGTGGAGCAGCATC-3'     | 8.1, 8.2       |
| V $\alpha$ 9  | HAP 36 | 5'-ATCTCAGTGTGTGATAATA-3'        | 9.1            |
| V $\alpha$ 10 | HAP 58 | 5'-ACCCAGCTGGTGGAGCAGAGCCCT-3'   | 10.1           |
| V $\alpha$ 11 | HAP 02 | 5'-AGAAAGCAAGGACCAAGTGT-3'       | 11.1           |
| V $\alpha$ 12 | PGA 5  | 5'-CAGAAGTAACCTAAGCGCAGACT-3'    | 12.1           |
| V $\alpha$ 13 | AB 11  | 5'-GCTTATGAGAACACTGCGT-3'        | 13.1           |
| V $\alpha$ 14 | AB 21  | 5'-GCAGCTTCCTCCAGCAAT-3'         | 14.1           |
| V $\alpha$ 15 | AC 24  | 5'-AGAACCTGACTGCCAGGAA-3'        | 15.1           |
| V $\alpha$ 16 | AE 212 | 5'-CATCTCCATGGACTCATATGA-3'      | 16.1           |
| V $\alpha$ 17 | AF 211 | 5'-GACTATACTAACAGCATGT-3'        | 17.1           |
| V $\alpha$ 18 | AC 9   | 5'-TGTCAGGCAATGACAAGG-3'         | 18.1           |
| C $\alpha$    | PGA 5  | 5'-AATAGTTCGAGACACTTGCTACTGGA-3' | C $\alpha$     |

The size of amplified products using 5' V $\alpha$  and 3' C $\alpha$  primers ranged from about 320 to 410 base pairs.



FIG. 1 Brain TCR amplification of MS patient 1. Lanes a: control brain cDNA; b, MS parietal region brain cDNA; c, MS occipital region brain cDNA; d, PGA5, a full-length TCR  $\alpha$  cDNA<sup>18</sup>. Complementary DNA (2  $\mu$ l) was combined in a 100- $\mu$ l reaction volume, with 1 unit of DNA Taq polymerase (Perkin Elmer-Cetus), 10  $\mu$ l 10 $\times$  reaction buffer, 50  $\mu$ M each of deoxynucleoside triphosphates, and 1  $\mu$ M of each primer. The PCR profile used was: denaturation 95 $^{\circ}$ C for 60 s, annealing 45 $^{\circ}$ C for 60 s and extension 72 $^{\circ}$ C for 60 s, for 35 cycles on a DNA Thermal Cycler (Perkin Elmer-Cetus). One tenth of each sample was independently run in a 4% Nusieve agarose gel (Fmc) and an appropriate size fraction was excised from the gel. The agarose piece was frozen and thawed 3 times, and 2  $\mu$ l supernatant directly reamplified with the same primers for an additional 25 cycles. A 500-bp actin fragment was successfully amplified from brain cDNA (lanes a-c), but not from the PGA5 control (lane d) using the following primers: 5'-ACGAAGACGGACACCGCCCTCG-3', 5'-CACGTTGTGGGTGACGCGTC-3'. V $\alpha$  and C $\alpha$  transcripts were amplified from both MS brain cDNA and PGA 5 templates, but not from the control MS brain cDNA with primers AB 13-14 (5'-CAGAAGTAACCTGCGCAGACT-3', 5'-TTGGGATCCAGACACAGAAAGTACTGTC-3'), which include PstI and BamHI restriction sites and define a 286-bp fragment of the V $\alpha$  12.1 region gene; and AB 41-42 (5'-CAGAACCCTGACCCCTGCGGTGAC-3', 5'-GTGTCCACAGTTTAGGTTCTGTATCTGT-3'), which includes a SalI site and defines a 340-bp fragment of the C $\alpha$  region transcript) respectively. Note that rearranged TCR $\alpha$  sequences could be amplified from cDNA of the MS brain prepared from the occipital region (lane c) using the V $\alpha$  12.1 primer AB 13 and C $\alpha$  primer AB 42.

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MS Br2  
MS Br3  
CB4  
CB5  
CB6

Experimer  
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MS Br2  
MS Br3  
CB4  
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TABLE 2 T-cell receptor V $\alpha$  expression in brain plaques of multiple sclerosis patients

|                         | V $\alpha$ 1 | V $\alpha$ 2 | V $\alpha$ 3 | V $\alpha$ 4 | V $\alpha$ 5 | V $\alpha$ 6 | V $\alpha$ 7 | V $\alpha$ 8 | V $\alpha$ 9 | V $\alpha$ 10 | V $\alpha$ 11 | V $\alpha$ 12 | V $\alpha$ 13 | V $\alpha$ 14 | V $\alpha$ 15 | V $\alpha$ 16 | V $\alpha$ 17 | V $\alpha$ 18 | V $\beta$ 8 | Actin   |
|-------------------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|-------------|---------|
| <b>Experiment no. 1</b> |              |              |              |              |              |              |              |              |              |               |               |               |               |               |               |               |               |               |             |         |
| MS Br1                  | 383          | 4,640        | 760          | 520          | 240          | 850          | 826          | 1,566        | 450          | 45,860        | 5,430         | 36,380        | 3,618         | 367           | 280           | 289           | 226           | 442           | 170         | 104,450 |
| MS Br2                  | 140          | 824          | 523          | 310          | 830          | 415          | 660          | 23,200       | 1,750        | 29,630        | 623           | 49,125        | 456           | 220           | 317           | 12,460        | 3,572         | 338           | 280         | 79,120  |
| MS Br3                  | 638          | 313          | 276          | 410          | 817          | 1,520        | 210          | 15,860       | 16,310       | 21,200        | 838           | 2,050         | 302           | 225           | 462           | 3,633         | 482           | 470           | 630         | 58,358  |
| C Br4                   | 235          | 1,100        | 135          | 115          | 285          | 7,300        | 427          | 960          | 1,036        | 317           | 560           | 726           | 485           | 278           | 466           | 630           | 545           | 830           | 900         | 65,996  |
| C Br5                   | 580          | 875          | 180          | 490          | 110          | 846          | 160          | 324          | 780          | 120           | 344           | 138           | 762           | 755           | 876           | 860           | 715           | 570           | 860         | 66,393  |
| C Br6                   | 137          | 290          | 133          | 530          | 836          | 640          | 910          | 110          | 140          | 350           | 670           | 1,030         | 1,095         | 2,000         | 437           | 775           | 240           | 330           | 710         | 139,337 |
| <b>Experiment no. 2</b> |              |              |              |              |              |              |              |              |              |               |               |               |               |               |               |               |               |               |             |         |
| MS Br1                  | 1,650        | 3,956        | 1,450        | 790          | 547          | 545          | 1,170        | 343          | 1,856        | 32,870        | 513           | 12,978        | 866           | 868           | 3,190         | 280           | 1,048         | 1,127         | 440         | 38,593  |
| MS Br2                  | 967          | 340          | 1,419        | 1,575        | 3,866        | 2,837        | 1,848        | 13,373       | 2,974        | 17,337        | 1,550         | 33,020        | 1,487         | 1,072         | 3,148         | 17,968        | 1,446         | 980           | 1,338       | 32,460  |
| MS Br3                  | 666          | 726          | 1,198        | 790          | 1,769        | 258          | 576          | 35,270       | 18,990       | 19,138        | 948           | 2,690         | 587           | 880           | 815           | 945           | 946           | 1,570         | 630         | 22,415  |
| C Br4                   | 1,507        | 660          | 1,740        | 1,790        | 553          | 706          | 4,540        | 4,410        | 1,333        | 584           | 919           | 765           | 860           | 206           | 590           | 713           | 2,748         | 526           | 864         | 31,285  |
| C Br5                   | 896          | 1,670        | 2,370        | 5,000        | 2,826        | 418          | 862          | 8,175        | 2,048        | 1,307         | 1,734         | 836           | 737           | 1,040         | 2,097         | 2,925         | 1,025         | 5,276         | 4,478       | 33,018  |
| C Br6                   | 883          | 1,727        | 716          | 865          | 610          | 1,334        | 9,514        | 1,033        | 1,256        | 1,130         | 636           | 170           | 4,636         | 1,300         | 1,930         | 1,167         | 764           | 5,915         | 370         | 29,451  |
| PBL(PHA)                | 9,434        | 19,464       | 8,288        | 18,434       | 18,820       | 10,483       | 12,800       | 14,886       | 13,980       | 23,040        | 11,448        | 16,968        | 16,536        | 17,750        | 30,512        | 16,544        | 21,132        | 19,732        | ND          | ND      |

Samples were taken from brain plaques of three patients with chronic progressive MS, and three controls (non-MS). Total RNA and cDNA (from 5  $\mu$ g RNA) were prepared according to standard procedures<sup>23</sup>. Control cDNA was also prepared from 1  $\mu$ g RNA isolated from a pool of peripheral blood lymphocytes from five different individuals, stimulated with 3  $\mu$ g ml<sup>-1</sup> of PHA. cDNAs were amplified with TCR V $\alpha$ -C $\alpha$  or actin primers for 40 cycles in the presence of 10  $\mu$ M of  $\alpha$ -[<sup>32</sup>P]dATP (Amersham). Samples were analysed by gel electrophoresis with ethidium bromide to identify the specific fragment band (italic figs when a band was clearly visible). After electrophoretic separation, bands were excised and incorporation of radiolabel was determined. Where TCR rearranged bands were absent, an agarose fragment 200-600 bp was excised and counted. An actin band was visualized in all the amplified brain cDNA. Results are expressed in counts per min. All TCR 5' primers amplify TCR sequences from germ line DNA using a specific 3' V $\alpha$  primer for each family. We have detected V-C $\alpha$  rearrangements of all TCR V gene members in a variety of activated T cells including single rearrangements of specific V $\alpha$  members in T-cell clones reactive to pertussis toxin, to *Borrelia burgdorferi*, as well as the Jurkat T cell line, and rearrangements of all V $\alpha$  members in pooled T cells stimulated by PHA (line 7, experiment 2). ND, Not determined.

on cDNA isolated from the parietal and occipital brain regions from one MS patient, and from the occipital brain region of one control (non-MS) individual, are shown in Fig. 1. Actin sequences were coamplified together with the V $\alpha$  12.1 gene to monitor the integrity of the cDNAs. Actin could be amplified from the brain cDNAs (500 base pair (bp) fragment (actin primer: lanes a-c) but not from PGA5, a full-length cDNA clone that contains the V $\alpha$  12.1 segment<sup>18</sup> (lane d). A smaller PCR product corresponding to the V $\alpha$  12.1 gene was detected in the patient but not in the control sample (282-bp fragment; primer AB13-AB14: lanes b, c and d). To ensure that only the V $\alpha$  12.1 family was amplified, genomic and brain cDNA PCR products were analysed using restriction endonucleases and were consistent with the known restriction map for V $\alpha$  12.1. When colonies containing cloned V $\alpha$  PCR products from MS brain cDNA were screened with a V $\alpha$  12.1 region probe, about 20% were positive. DNA from several of these colonies was sequenced, and was found to be identical with the V $\alpha$  12.1 sequence<sup>18</sup>. Thus, the TCR V $\alpha$  12.1 restriction fragment-length polymorphism recently associated with MS susceptibility<sup>15</sup>, must be in a sequence flanking the V $\alpha$  12.1 gene. Specific regulation of the  $\alpha$ -TCR gene by 3' cis-acting enhancers, was recently demonstrated<sup>19</sup>.

These experiments indicated that PCR could amplify the receptor transcripts from post-mortem brain samples, starting from nanograms of total RNA without the necessity of *in vitro* expansion of T cells. Similarly, C $\alpha$  sequences were amplified from MS brain cDNAs, but not from the control sample (primers AB41-AB42: lanes b and c, Fig. 1). A subsequent amplification was carried out (primers AB13-AB42: lanes a, b, c and d) using a 5' primer complementary to the V $\alpha$  12.1 and a 3' primer complementary to the C $\alpha$  TCR regions. These primers, which can amplify only rearranged TCR transcripts, amplified a product of ~680 bp from both the positive control PGA5 sample and cDNA from the occipital region of the MS brain (primers AB13-AB42: lanes c and d) but not from control brain cDNA or cDNA from the parietal region of the MS brain (primers AB13-AB42: lanes a and b). The V $\alpha$  and C $\alpha$  amplifications from the MS brain parietal region most probably represent transcripts from unrearranged chromosomes, as has been found in other cDNA libraries from T cell lines<sup>20</sup>. No PCR product was observed using primers corresponding to the V $\beta$  8 family, even though this TCR V $\beta$  region was recently reported to be associated with susceptibility to MS (ref. 13).

To substantiate that the DNA produced by PCR was an authentic amplified product of rearranged TCR genes, the PCR products were sequenced using the dideoxy chain termination

method<sup>21</sup> after double screening of colonies with V $\alpha$  and C $\alpha$  probes. Only two different J regions were seen in the 25 clones examined. Both were different from the PGA5 J $\alpha$  sequence, ruling out the possibility of a 'carryover' contamination. Eleven clones contained the J $\alpha$ O family found in clone HAP 41 (ref.

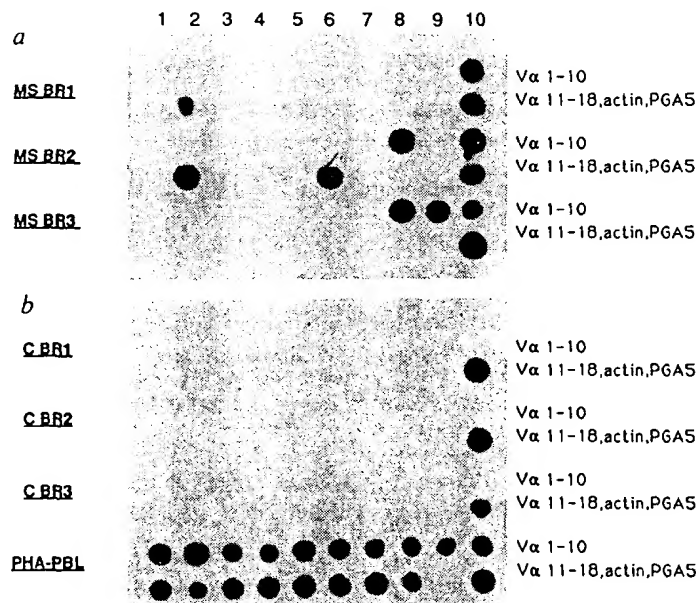


FIG. 2 Detection of PCR-amplified products by dot-blot hybridization using a C $\alpha$  probe. a, MS brain (MS BR) amplifications; b, control brain (C BR) and PHA-PBL amplifications.

METHODS. The amplified product (10  $\mu$ l) was dot-blotted onto transfer membranes (GeneScreen Plus, NEN) after denaturation with 0.4 M NaOH-25 mM Na<sub>2</sub>EDTA. Actin PCR products served as negative controls and PGA5 as the positive control. Filters were fixed under ultraviolet light and prehybridized for 3 h at 42 °C in 5 $\times$ SSPE/5 $\times$  Denhardt's solution/salmon sperm DNA (100  $\mu$ g ml<sup>-1</sup>)/0.1% SDS and hybridized overnight at 42 °C with 1 $\times$  10<sup>6</sup> c.p.m. per ml of <sup>32</sup>P-kinked probe (5'-AATATCCAGAACCCCTGACCC-3'). Filters were washed in 1 $\times$ SSPE/0.1% SDS at room temperature, twice for 20 min; and then in 0.1 $\times$ SSPE/0.1% SDS at 45 °C twice for 10 min. Kodak XAR-5 film with Dupont Lightning-Plus intensity screens were used for autoradiography at -70 °C for 30 min. Signals correlate with the presence of a specific band in the ethidium bromide-stained agarose gel and with high [<sup>32</sup>P]dATP incorporation during amplification (Table 2).

22) and 14 clones had a previously undescribed  $\alpha$  sequence (GGGTACCGAGATGACGAACCCACCTTTGGGACAGG-CACTCAGCTAAAAGTGGAACTC).

We next asked whether there was a diverse or limited usage of TCR  $V\alpha$  gene expression in MS brain lesions. To analyse the use of TCR  $V\alpha$  in MS brains, we synthesized 5' PCR primers for the 18 different  $V\alpha$  families<sup>22,23</sup> (Table 1). Optimal conditions for amplification were ascertained using each of the 5' primers (Table 1) in combination with a specific 3'  $V\alpha$  primer for each TCR  $V\alpha$  family, on genomic DNA (data not shown) and with a common  $C\alpha$  primer (AB 51) on reverse-transcribed RNA isolated from phytohaemagglutinin (PHA)-stimulated peripheral blood lymphocytes (Table 2). The results from amplification of MS brain cDNA, using 5'  $V\alpha$  primers and the common 3'  $C\alpha$  primer AB 51 in the presence of  $\alpha$ -[<sup>32</sup>P]dATP, showed that in each brain only a few TCR  $V\alpha$  gene families are preferentially rearranged and transcribed (Table 2). Visualization of a specific band for actin or rearranged TCR after gel electrophoresis of the amplified product, is indicated by italics in Table 2. In general, high [<sup>32</sup>P]dATP incorporation was reproducibly detected in regions of the gel where bands were visualized.

These results were further confirmed by dot blot of the PCR products and hybridization with a radiolabelled oligonucleotide corresponding to a TCR- $C\alpha$  sequence, 5' from the common  $C\alpha$  primer (Fig. 2). A clear signal in the dot-blot assay correlates with the presence of a band staining with ethidium bromide in the agarose gel. Products of amplifications with actin primers were used as a negative control; amplification products of clone PGA5 with TCR  $V\alpha$  and  $C\alpha$  primers were used as a positive control. Expression of TCR  $V\alpha$  transcripts is operationally defined by (1) the presence of a rearranged  $V\alpha$ - $C\alpha$  band visualized with ethidium bromide, and (2) a signal on dot-blot hybridization of the amplified  $V\alpha$ - $C\alpha$  gene with a TCR  $C\alpha$  probe, 5' from the  $C\alpha$  PCR primer. Thus in MS brain 1, TCR  $V\alpha$  10 and 12 were rearranged; TCR  $V\alpha$  8, 10, 12 and 16 were rearranged in MS brain 2, and  $V\alpha$  8, 9 and 10 were rearranged in MS brain 3. No  $V\alpha$  transcripts could be amplified from control brain cDNA, although actin could be amplified. Each of 18 TCR  $V\alpha$  rearranged genes were amplified from PHA-stimulated pooled lymphocytes. The  $V\alpha$  10 family was detected in all three MS samples, suggesting that this TCR might be responding to a major epitope of an antigen involved in the pathogenesis of MS. Further experiments will be needed to confirm this hypothesis.

Our results may prove to have therapeutic implications. Studies on TCR gene expression in experimental allergic encephalomyelitis (EAE), a prototypic animal model for autoimmunity induced by T cells, have shown the predominant use of certain TCR  $\alpha$  and  $\beta$  products in the immune response to myelin basic protein<sup>24-27</sup>. Treatment of mice *in vivo* who have EAE with monoclonal antibody specific to the predominant TCR V-region product reverses paralytic disease<sup>24</sup>. In addition, it is possible to prevent EAE by immunization with inactive encephalitogenic T cells<sup>28</sup> or with synthetic peptides from the CDR2 and CDR3 TCR V regions that are rearranged in the encephalitogenic clones<sup>29,30</sup>. Elucidation of TCR expression in the brain may help in the design of similar treatments in MS patients. □

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## Nitric oxide as an inhibitory non-adrenergic non-cholinergic neurotransmitter

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INHIBITORY non-adrenergic non-cholinergic (NANC) nerves are thought to be important in the autonomic innervation of the gastrointestinal tract and other organ systems. The nature of their neurotransmitter is still debated. Speculation that nitric oxide (NO), formed from L-arginine in neuronal structures<sup>1</sup> and other cells<sup>2</sup>, could act as a neurotransmitter, is not yet supported by demonstration of its release upon nerve stimulation. Using a superfusion bioassay, we report the release of a vasorelaxant factor upon stimulation of the NANC nerves<sup>3</sup> in the canine ileocolonic junction. Several pieces of evidence, including the selectivity of the bioassay tissues, chemical instability, inactivation by superoxide anion and haemoglobin, inhibition by N<sup>G</sup>-nitro-L-arginine (L-NNA)<sup>4</sup> and potentiation by L-arginine all indicated that NO accounted for the biological activity of this transferable NANC factor.

The canine ileocolonic junction (ICJ) was isolated, superfused with Krebs-Ringer solution and the effluent superfused de-endothelialized rings of rabbit aorta<sup>5</sup>, arranged either in a cascade<sup>6,7</sup> (Fig. 1) or in parallel. Upon electrical stimulation (ES; 16 Hz, 2 ms) or infusion of the nicotinic receptor agonist 1,1-dimethyl-4-phenylpiperazinium (DMPP), ICJ tissue released a vasodilator activity, causing 22.4 ± 3.2% (*n* = 12) and 26.3 ± 3.9% (*n* = 9) relaxation of the top tissue respectively. The release of the factor was frequency-dependent and its activity declined (39 ± 8%, *n* = 8, ES; 41 ± 15%, *n* = 4, DMPP) during passage down the cascade. As atropine and guanethidine were present and as the detector tissues failed to relax to acetylcholine (ACh) and noradrenaline (Figs 1 and 2), the transferable factor is indeed non-adrenergic and non-cholinergic. Furthermore, blockade of nerve conductance with tetrodotoxin abolished the release of the vasodepressant factor to both stimuli (Fig. 2), indicating that neuronal structures were activated. Non-selective stimulation of other cell populations<sup>2</sup> or microorganisms therefore seems unlikely.

The instability of the vasodilator factor was compatible with NO<sup>7,8</sup>, as confirmed by the injection of authentic NO (Fig. 1). Moreover, haemoglobin (Hb), known to trap NO avidly<sup>9</sup>, eliminated the biological activity, whereas dilatation, due to NO

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